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Effects of Zafirlukast on the Function of Human Polymorphonuclear Neutrophil Leukocytes in Asthmatic Patients: A Prospective, Controlled, In Vitro Study

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ABSTRACT

Background: Reactive oxygen species (ROSs) play an important role in the pathogenesis of asthma, and oxidative stress contributes to the initiation and worsening of inflammatory respiratory disorders (eg, asthma). Thus, antioxidant drugs may have a role in reducing or preventing damage in asthma.

Objective: The aim of the study was to investigate the antioxidant effect of zafirlukast, a leukotriene receptor antagonist, in asthma.

Methods: This prospective, controlled, in vitro study was conducted at King Khalid University Hospital, Riyadh, Saudi Arabia. The generation of ROSs by polymorphonuclear neutrophil leukocytes (PMNs) in patients with mild to moderate asthma (forced expiratory volume in 1 second [FEV_1], >70% of the predicted value) and healthy volunteers was assessed using chemiluminescence (CL) with phorbol 12-myristate 13-acetate (PMA) and opsonized zymosan (OPZ) in the presence of different concentrations of zafirlukast (1.25–60 μg/mL). The xanthine/xanthine oxidase (X-XOD) reaction was used to test the scavenging effect of the drug.

Results: Six asthmatic patients (4 women, 2 men; mean age, 30.8 years; mean FEV_1, 82.5% of the predicted value) and 8 healthy volunteers (4 women, 4 men; mean age, 28.8 years) were enrolled. A dose-dependent inhibition of the CL response was observed in both groups. However, patients with asthma required higher concentrations of zafirlukast to achieve an inhibitory effect similar to that in healthy controls. This difference was significant at concentrations of 20 to 60 μg/mL (all, $P \leq 0.05$). When PMNs were challenged with OPZ, inhibition was also dose dependent in controls at all concentrations (all, $P \leq 0.05$), but the inhibitory effect was not significant in the asthmatic patients at any concentration. The difference in the inhibitory effect between the 2 groups was significant

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at 30, 40, and 60 μg/mL ($P < 0.02$, <0.01, and <0.01, respectively). The mean (SEM) viability of the PMNs in the healthy controls was significantly affected only at the highest concentration compared with the control saline dose (86.5% [5.8%] vs 97.0% [8.%]; $P < 0.05$). No scavenging effect of zafirlukast was found using the X-XOD system. Incubating PMA-stimulated cells with zafirlukast (5 and 10 μg/mL) for 10 minutes to 1 hour significantly increased the inhibitory effect of the drug by 15% to 46% (all, $P < 0.001$). When zafirlukast was tested for reversibility of its inhibitory effect on ROS production, its action was found to be irreversible at a concentration of 30 μg/mL ($P < 0.001$) and partially reversible at 60 μg/mL compared with the baseline saline control.

Conclusions: Zafirlukast inhibited ROS generation by PMNs in a dose-dependent manner in asthmatic patients and healthy subjects. However, asthmatic patients required much higher concentrations compared with controls. The incubation of the stimulated cells with zafirlukast increased the inhibitory effect. This finding suggests that the therapeutic effect of zafirlukast in asthma may be in part related to its antioxidant action. (*Curr Ther Res Clin Exp.* 2005; 66:279–293) Copyright © 2005 Excerpta Medica, Inc.

Key words: zafirlukast, polymorphonuclear leukocytes, asthmatic patients, reactive oxygen species, healthy volunteers, chemiluminescence.

INTRODUCTION

Bronchial asthma, which is most accurately thought of as a syndrome consisting of a common pathway of injury from a variety of insults, can be mediated through multiple mechanisms of inflammation and repair.[1] Neutrophils, eosinophils, and other cells involved in the inflammatory response can generate large amounts of free radicals, which can be measured in exhaled gas. Hanazawa et al[2] reported a significant increase in exhaled free radical metabolites in patients with mild asthma who were not treated with corticosteroids. Neutrophils with altered function have been found in blood from asthmatic patients before and after antigen challenge. These alterations include decreased density, increased complement receptor type 3 expression, altered response to regulating agents, generation of oxygen metabolites, and increased leukotriene (LT) production and secretion.[3]

Stimulation of human leukocytes by various agents, including phagocytic stimuli, results in the synthesis of the bioactive arachidonic acid–derived LTs. The major arachidonic acid–derived metabolites synthesized by polymorphonuclear neutrophil leukocytes (PMNs) are LTs A4 and B4. The free arachidonic acid generated by phospholipase A_2 can be released to the extracellular compartment, where it is utilized in LT biosynthesis by agonist-stimulated PMNs. In pathologic situations, such as bronchial asthma, the efficient utilization of accumulated arachidonic acid results in increased production of LTs.[4]

The cysteinyl LTs C4 and D4 (cys LTC4 and LTD4) have been found to be potent bronchoconstrictors in guinea pig airways in vitro and in vivo and to

cause isolated human bronchi to contract.[5] LTC4, LTD4, and LTE4 have been found to cause tissue edema.[6] It has also been observed that LTC4 and LTD4 may stimulate mucus secretion in isolated animal[7] and human[8] airways. More recently, additional effects with potential relevance to the role of cys LTs in asthma and pulmonary inflammation have been reported. Increased infiltration of eosinophils into the airway mucosa of asthmatic patients have been observed after inhalation of LTE4 and LTD4.[9] Urinary excretion of LTE4 has been found to be increased during an acute attack of asthma[10] and after challenge with allergens, exercise, or aspirin ingestion.[11] In a guinea pig trachealis model, Weiss and Bellino[12] found a statistical correlation between exogenous LTD4 and reactive oxygen species (ROS) production that resulted in acquired hyperreactivity to histamine in airway smooth muscle. This effect was blocked by an LT antagonist (FPL 55712) and, unexpectedly, by superoxide dismutase, suggesting that LTD4 stimulated the release of ROSs, which were mainly derived from PMNs and/or mast cells.

Pharmacotherapy for asthma, which has remained essentially unchanged over the past 3 decades, consists of glucocorticoids, β_2-agonists, and theophylline. Based on the involvement of LTs in the production of bronchoconstriction, antileukotrienes, and in particular the LT receptor antagonists, offer an important alternative class of drug therapy for asthma.[13] These drugs have some advantages over bronchodilators used to treat bronchial asthma because the disease is associated with an inflammatory reaction.[14] Zafirlukast is a potent and selective cys LT receptor antagonist that has been approved by the US Food and Drug Administration for the prophylactic treatment of asthma in patients aged ≥12 years.[15] The drug causes significant attenuation of the early and late responses to inhalational antigens and reduces the bronchial hyperresponsiveness to histamine that typically accompanies antigen inhalation.[16] Zafirlukast complements the anti-inflammatory effects of corticosteroids and has been studied in combination with corticosteroids as an alternative to high-dose inhalational corticosteroids.[17] Despite these actions of zafirlukast, PubMed and Ovid searches (key words: *asthma, reactive oxygen species,* and *zafirlukast*; years: 1996–2005) revealed that the effect of zafirlukast on the function of PMNs isolated from asthmatic patients has not yet been studied.

The aim of this in vitro study was to investigate whether zafirlukast affects the production of ROSs from PMNs obtained from asthmatic patients and healthy subjects.

PATIENTS AND METHODS

Inclusion and Exclusion Criteria

Asthmatic patients and healthy volunteers aged 20 to 45 years were recruited for this prospective, controlled, in vitro study. The volunteers were recruited from a pool of blood donors at King Khalid University Hospital, Riyadh, Saudi Arabia. Patients were eligible if they were clinically stable and known to have

mild to moderate asthma, defined as forced expiratory volume in 1 second (FEV_1) >70% of the predicted value; and reversible airway disease, defined as a ≥15% increase in FEV_1 after bronchodilator inhalation. Asthmatic patients receiving continuous maintenance therapy were excluded. Subjects were excluded if they had a history of recent infection, had any other medical problems (determined by subject interview), or were smokers. Patients were instructed not to use any medications for at least 24 hours before the collection of blood samples.

All individuals provided oral informed consent to participate in the study. The study was approved by the ethics committee of King Khalid University Hospital.

Peripheral venous blood was drawn from all study participants and transferred to heparinized tubes.

Material Preparation

Polymorphonuclear Neutrophil Leukocyte Separation

PMNs were separated using a neutrophil isolation medium (Cardinal Associates Inc., Santa Fe, New Mexico). Five to 7 mL of heparinized blood was layered over 4 mL of the neutrophil isolation medium in a 15-mL tube, which was centrifuged (Heraeus-Christ GmbH, Osterode, Germany) at 400*g* for 30 minutes at room temperature. The leukocyte-rich plasma was removed using a Pasteur pipette and transferred to a 15-mL conical centrifuge tube. The tube was filled with phosphate-buffered saline (PBS) and centrifuged at 350*g* for 10 minutes at room temperature. The residual erythrocytes were lysed using 2 mL of lysing buffer (E-lyse, Cardinal Associates Inc.), vortexed to resuspend the pellets, and then centrifuged at 250*g* for 10 minutes at room temperature. The supernatant was discarded and the sediment was suspended in 1 mL of 5% fetal calf serum. The cells were counted and adjusted to a concentration of 5×10^6 cells/mL.

Reagents

Various stimulants made it possible to define the signal transduction pathways and the involvement of other cellular functions, such as phagocytosis and degranulation. Clinically, phorbol 12-myristate 13-acetate (PMA) was used to monitor cellular defects, and opsonized zymosan (OPZ) was used to detect defects in immunologic functions.[18]

Phosphate-Buffered Saline Solution

PBS was dissolved in distilled water to the following concentrations: sodium chloride 0.14 M, potassium chloride 2.7 mM, disodium hydrogen orthophosphate 12 mM, potassium dihydrogen phosphate 1.5 mM, calcium chloride 0.9 mM, and magnesium chloride 0.49 mM.

Luminol

Luminol (no. 1243-216, LKB-Wallac Company, Turku, Finland) was dissolved in dimethyl sulfoxide to give a concentration of 1.77 mg/mL, which was further diluted in PBS to 17.7 µg/mL prior to use.

Phorbol Myristate Acetate Solution

PMA was dissolved in dimethyl sulfoxide to give a stock solution of 2 mg/mL. The solution was stored at –20°C until use. It was further diluted with PBS to 20 ng/mL prior to use.

Opsonization of Zymosan

Particulate zymosan was washed twice and resuspended in PBS, and its concentration was adjusted to 12.5 mg/mL. To opsonize the drug, 900 µL of zymosan suspension was incubated at 37°C for 30 minutes with 100 µL of autologous human serum obtained from healthy individuals. It was then washed with PBS and resuspended in PBS to give a final concentration of 1.25 mg/mL.

Zafirlukast

To each 2 mg of zafirlukast powder 6 µL of 1N sodium hydroxide was added until the mixture appeared pasty. Then 100 µL of polyethylene glycol (molecular weight, 400) was added. This pastelike material was warmed at ~50°C until a clear solution was obtained. PBS (pH 7.4) was added (in increments of 200 µL to avoid precipitation) to make 2 mL of solution. The stock solution (1 mg/1 mL) was further diluted to the required concentration (range, 1.25–60 µg/mL). For example, to prepare the 60-µg/mL concentration, 60 µL of the stock solution, which contained 60 µg, was transferred to a tube, and 940 µL of PBS was added to reach the required volume (1 mL).

Assessments

Chemiluminescent Assay

The chemiluminescent assay was performed using a luminometer (model 1251, LKB-Wallac Company), which is a bench-top, computer-controlled instrument that monitors phagocytic events in real time. The luminometer was coupled with a monitor and a printer. Disposable polystyrene cuvettes and micropipettes with disposable tips for dispensing 100- to 1000-µL volumes were used.

The effect of zafirlukast on the generation of ROSs, as assessed using peak chemiluminescence (CL) as described previously,[19] was determined by the addition of different concentrations of the drug to isolated PMNs that were stimulated by PMA or OPZ.

Various reagents were placed into quadruplet cuvettes using a pipette. The first tube was the baseline, containing 700 µL PBS, 200 µL luminol, and 100 µL PMNs. The second was the control tube containing 500 µL PBS, 200 µL luminol, 100 µL PMNs, and 200 µL of a stimulant (PMA or OPZ). The third and fourth tubes contained 400 µL PBS, 200 µL luminol, 100 µL PMNs, 100 µL zafirlukast, and 200 µL of a stimulant (PMA or OPZ). The cuvette contents were mixed gently, and the light emission was recorded in millivolts. The assay temperature was maintained at 37°C. Each sample was measured over a period of 30 minutes. Using a computer, the results were plotted on a graph, with the *y*-axis representing the light intensity (mV) and the *x*-axis representing the time (minutes).

Cell-Free Xanthine/Xanthine Oxidase System

The xanthine/xanthine oxidase (X-XOD) reaction was used to generate superoxide, as described by Gionchetti et al.[20] The catalyzed reaction of XOD on xanthine was induced by incubating 0.05 U/mL of XOD in PBS (pH 7.4) containing 0.1 mmol/L EDTA, 50 mmol/L xanthine, and 10^{-4} mol/L luminol. The different concentrations of the drug were incubated with xanthine and luminol for 5 minutes before XOD was added. Superoxide was measured using CL.

Polymorphonuclear Leukocyte Viability

Using concentrations of 30 and 60 µg/mL, the cytotoxicity of zafirlukast on PMNs was assessed in healthy controls. PMNs were incubated in zafirlukast for 10 minutes to 1 hour at 37°C. The percentage of viable cells was estimated using the trypan blue (0.2% w/v) exclusion test (ie, the cells that absorbed the dye were considered nonviable).

Effect of Zafirlukast Incubation on Polymorphonuclear Neutrophil Leukocytes

The effect of zafirlukast 5 and 10 µg on ROS generation was tested at 0, 10, 30, and 60 minutes of zafirlukast incubation at 37°C.

Reversibility of Zafirlukast Action

To investigate whether zafirlukast would have a permanent effect on PMNs, zafirlukast 30 and 60 µg were incubated for 45 minutes with isolated PMNs and washed several times with PBS. The CL of unwashed and washed samples was recorded in the presence or absence of zafirlukast.

Statistical Analysis

The nonparametric Mann-Whitney *U* test was used to compare results from the asthmatic patients with those from healthy controls. A P value <0.05 was considered statistically significant. A power analysis was not performed. All data are presented as mean (SEM) of the peak CL.

RESULTS

Study Population

Six asthmatic patients (4 women, 2 men; mean age, 30.8 years; mean FEV_1, 82.5% of the predicted value) and 8 healthy volunteers (4 women, 4 men; mean age, 28.8 years) were recruited for the study.

Polymorphonuclear Neutrophil Leukocyte Chemiluminescence Response

When PMNs were challenged with PMA in the presence of increasing concentrations of zafirlukast (1.25–60 µg/mL), significant dose-dependent inhibition of CL response was observed with concentrations of 5 to 60 µg/mL in asthmatic patients (all, $P \leq 0.05$) and with concentrations of 2.5 to 60 µg/mL in healthy controls (all, $P < 0.001$) compared with the baseline saline control dose (**Table I**).

Table I. Effects of zafirlukast on phorbol myristate acetate–induced peak chemiluminescence responses of polymorphonuclear neutrophil leukocytes isolated from patients with mild to moderate asthma and healthy volunteers (control group).

Zafirlukast Concentration, µg/mL	Peak Chemiluminescence Response, Mean (SEM), mV		
	Asthmatic Patients (n = 6)	Healthy Volunteers (n = 8)	P*
0†	431.5 (57.6)	474.2 (31.5)	1.00
1.25	441.9 (58.2)	441.5 (31.0)	0.70
2.5	424.8 (65.3)	371.3 (28.6)‡	0.07
5	323.8 (39.9)§	254.1 (21.7)‡	0.07
10	270.3 (34.6)‡	168.9 (15.2)‡	0.09
20	230.4 (25.7)‡	142.8 (11.5)‡	0.05
30	203.8 (23.5)‡	132.4 (10.1)‡	0.05
40	165.2 (28.7)‡	97.7 (8.5)‡	0.01
60	139.9 (41.4)‡	55.5 (3.8)‡	0.006

*Between-group differences.
†Saline (baseline control).
‡$P < 0.001$ versus baseline control.
§$P < 0.05$ versus baseline control.

The inhibitory effect was significantly different between the 2 groups at 20 to 60 µg/mL (all, $P \leq 0.05$), with the highest inhibitory effect achieved with zafirlukast 60 µg/mL. The concentrations of zafirlukast required to inhibit peak CL by 50% in patients and controls were 25 and 5 µg/mL, respectively (**Figure 1**).

When OPZ was used as a stimulant, the inhibition was dose-dependent in healthy controls at all concentrations (all, $P \leq 0.05$), but the inhibitory effect was nonsignificant in the asthmatic group at all concentrations. The difference in the inhibitory effect between the 2 groups was significant at 30, 40, and 60 µg/mL ($P < 0.02$, <0.01, and <0.01, respectively) (**Table II** and **Figure 2**).

Polymorphonuclear Neutrophil Leukocyte Viability

We found significant mean (SEM) viability with the highest concentration of zafirlukast (60 µg/mL) compared with the baseline saline control dose (86.5% [5.8%] vs 97.0% [8.0%]; $P < 0.05$) (**Table III**).

Cell-Free System

Zafirlukast had no inhibitory effect on superoxide generation using the X-XOD reaction.

Effect of Incubation of Zafirlukast on the Chemiluminescence Response of Isolated Polymorphonuclear Neutrophil Leukocytes

Ten-minute incubation of PMA-stimulated cells with zafirlukast increased the inhibitory effect of the drug by 15% for the 5- and 10-µg/mL concentrations in

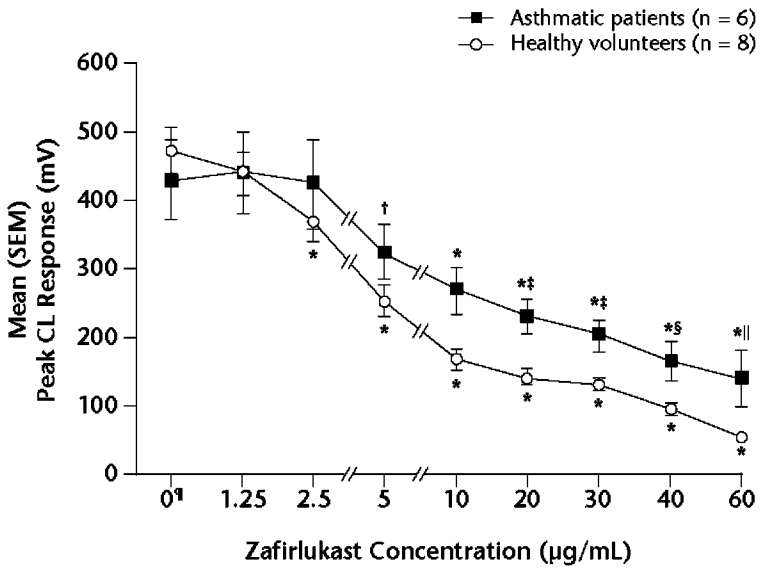

Figure 1. Effects of zafirlukast on the phorbol myristate acetate–induced chemiluminescence (CL) responses of polymorphonuclear neutrophil leukocytes isolated from patients with mild to moderate asthma and healthy volunteers (control group). *$P < 0.001$ versus baseline control; †$P < 0.05$ versus baseline control; ‡$P = 0.05$ versus control group; §$P = 0.01$ versus control group; ‖$P = 0.006$ versus control group; ¶Saline (baseline control).

Table II. Effects of zafirlukast on opsonized zymosan acetate–induced peak chemiluminescence responses of polymorphonuclear neutrophil leukocytes isolated from patients with mild to moderate asthma and healthy volunteers (control group).

Zafirlukast Concentration, µg/mL	Peak Chemiluminescence Response, Mean (SEM), mV		
	Asthmatic Patients (n = 6)	Healthy Volunteers (n = 8)	*P**
0†	881.8 (170.8)	587.5 (33.4)	0.81
1.25	998.0 (148.1)	519.9 (32.8)‡	0.26
2.5	1024.8 (157.5)	506.5 (32.0)‡	0.17
5	859.6 (161.4)	493.7 (31.5)§	0.17
10	829.8 (169.5)	482.7 (31.0)§	0.19
20	811.7 (155.6)	438.8 (30.7)§	0.11
30	777.1 (160.1)	330.1 (29.0)§	0.02
40	748.6 (148.5)	201.5 (20.3)§	0.01
60	821.5 (119.2)	78.3 (6.5)§	0.01

*Between-group differences.
†Saline (baseline control).
‡$P < 0.05$ versus baseline control.
§$P < 0.001$ versus baseline control.

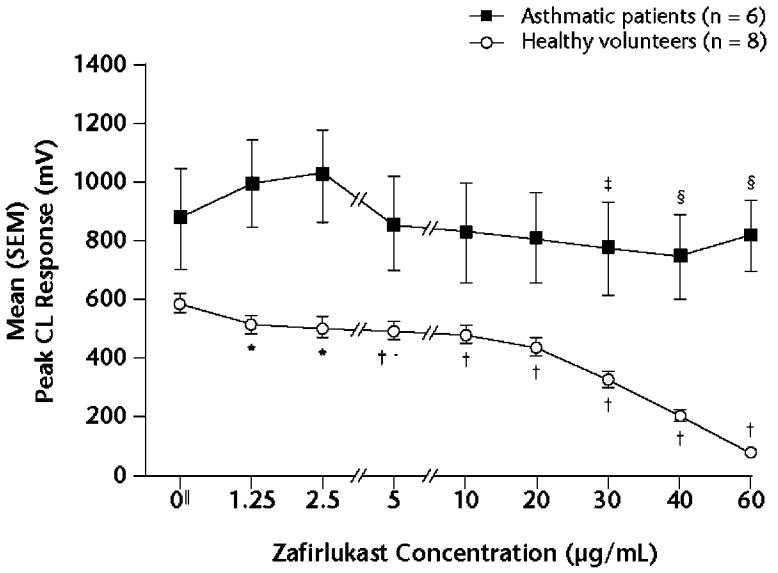

Figure 2. Effects of zafirlukast on the opsonized zymosan–induced chemiluminescence (CL) responses of polymorphonuclear neutrophil leukocytes isolated from patients with mild to moderate asthma and healthy volunteers (control group). *$P < 0.05$ versus baseline control; †$P < 0.001$ versus baseline control; ‡$P = 0.02$ versus control group; §$P = 0.01$ versus control group; ‖Saline (baseline control).

Table III. Effects of zafirlukast on the viability of polymorphonuclear leukocytes isolated from healthy volunteers (control group; n = 6). Values are presented as mean (SEM).

Zafirlukast Concentration, µg/mL	Viability, %
0*	97.0 (8.0)
30	95.2 (6.7)
60	86.5 (5.8)

*Saline (baseline control).
†$P < 0.05$ versus baseline control.

healthy controls (both, $P < 0.001$). Incubation for 30 minutes resulted in an increase in the inhibition of 30% to 35% for both concentrations, and the 1-hour incubation increased the inhibitory effect of the drug by 34% to 46% (all, $P < 0.001$) (**Table IV**).

Reversibility of the Inhibitory Effect of Zafirlukast

Irreversibility of zafirlukast action was significant at 30 µg/mL compared with baseline saline control ($P < 0.001$). Inhibition of the CL response remained almost unchanged (before washing, 70%; after washing, 65%). The action of a higher concentration (60 µg/mL) was found to be partially irreversible before and after cell washing (86.0% and 54.6% inhibition, respectively) (**Table V**).

DISCUSSION

The results of the present study suggest an inhibitory effect of the cys LT receptor antagonist zafirlukast on ROS production by PMNs. This effect was more evident in healthy volunteers compared with asthmatic patients. To explore the association between LTs and ROS production, we used the luminol-dependent CL method to examine the effect of zafirlukast on the capacity of isolated PMNs to generate ROSs. The results obtained in healthy controls showed that, without incubation, zafirlukast was associated with significant inhibition of both PMA- and OPZ-stimulated ROS generation in a concentration-dependent manner. In contrast, Braga et al[21] found that the incubation of different concentrations of zafirlukast with PMNs for only 10 minutes did not have a significant effect on the respiratory bursts induced by different stimuli (ie, *Candida albicans* and zymosan). However, prolonging the incubation for 1 hour resulted in a reduction in the respiratory bursts of PMNs in healthy volunteers at a concentration of 5×10^{-6} M (2.88 µg/mL) when they were stimulated with PMA but not zymosan. The difference between our findings and those of Braga et al[21] is probably related to the different concentrations of the drug used. In our study, the concentrations used ranged from 1.25 to 60 µg/mL, whereas the concentrations

Table IV. Effects of incubation with zafirlukast on phorbol myristate acetate–induced peak chemiluminescence responses of polymorphonuclear neutrophil leukocytes isolated from healthy volunteers (control group; n = 6).

Zafirlukast Concentration, µg/mL	Peak Chemiluminescence Response, Mean (SEM), mV			
	0 Min	10 Min	30 Min	60 Min
0*	474.5 (31.5)	429.3 (30.2)	352.4 (28.6)	289.1 (25.6)
5	254.4 (21.7)†	160.6 (14.5)†	61.7 (2.5)†	20.9 (1.6)†
10	169.0 (15.0)†	88.5 (7.3)†	19.9 (1.2)†	5.8 (0.7)†

*Saline (baseline control).
†$P < 0.001$ versus baseline control.

Table V. Reversibility of the effects of zafirlukast on phorbol myristate acetate–induced peak chemiluminescence responses of polymorphonuclear neutrophil leukocytes isolated from healthy volunteers (control group; n = 6).

Zafirlukast Concentration, μg/mL	Peak Chemiluminescence Response, Mean (SEM), mV		
	Unwashed	Washed	*P**
0†	450.5 (31.0)	390.6 (29.8)	0.007
30	135.2 (3.5)‡	135.6 (3.7)‡	0.87
60	63.1 (4.1)‡	177.2 (2.2)‡	0.23

*Unwashed versus washed.
†Saline (baseline control).
‡$P < 0.001$ versus baseline control.

used in the study by Braga et al[21] ranged from 5×10^{-9} to 5×10^{-6} M (0.0028–2.88 μg/mL), which were less than the therapeutic plasma drug concentration (0.25–10 μg/mL).[15] In the present study, significant inhibition of ROS production was observed at the lowest concentration of the drug used, without any incubation. However, incubation of different concentrations of zafirlukast for up to 1 hour was associated with an increase in the inhibitory effect of the drug, a finding that is consistent with those of Braga et al.[21]

In the present study, zafirlukast was associated with more inhibition of PMA-stimulated ROS production compared with that induced by OPZ. This finding agrees with that of Della Bianca et al,[22] who concluded that, depending on the receptor or combination of receptors activated, different signal transduction pathways might be involved in phagocytosis and the associated respiratory burst. Another study showed that, after activation of PMNs, significant amounts of LTC4 were released.[23] In addition, Raulf and Konig[24] found that stimulation of human PMNs with OPZ resulted in time- and dose-dependent releases of LTB4 and LTC4. On the other hand, priming of PMNs with PMA has been shown to increase 5-lipoxygenase kinase activity by translocation of 5-lipoxygenase to the nucleus and increases its capacity for phosphorylation.[25] Therefore, signal transduction pathways involved in the stimulation of PMA receptors will result in high 5-lipoxygenase production.[25]

In the present study, the PMNs of asthmatic patients were found to be more resistant to inhibition when stimulated with PMA compared with those of controls. A possible cause of this difference is that the PMNs of asthmatic patients might have increased production of ROSs[26] and LTs[3] compared with controls, and thus the asthmatic patients required higher doses of the drug to overcome the exaggerated response of PMNs to the stimulator. Another possible mechanism is that increased production of leukotrienes in asthmatic patients led to a decrease in their receptors on PMNs, which decreased the response of the cells to the drug.

In the present study, no statistically significant inhibition of PMNs was found after OPZ stimulation in asthmatic patients, whereas statistically significant inhibition was found in the controls. This difference could be due to the increase in complement receptor type 3, which is involved in clearing OPZ on PMNs in asthmatic patients.[3,27]

Cys LTs have 2 classes of G-protein–coupled receptors: cys LT1R and cys LT2R.[28] Both receptors are expressed on eosinophils and mast cells, whereas cys LT1R is expressed predominately on smooth muscle cells, lung fibroblasts, and neutrophils.[29] It has been suggested that LTD4 receptors on human bronchial smooth muscle are coupled to phospholipase D activation, yielding diacylglycerol and resulting in the activation of phosphokinase C.[30] Therefore, LTD4-induced contraction, at least in part, is independent of increases in Ca^{2+} release. It has also been suggested that during an asthmatic attack, eosinophils, macrophages, and mast cells produce cys LTs in the lung, which might activate cys LT1R in bronchial smooth muscle, leading to bronchoconstriction.[31] Activation of neutrophils leads to the generation of cys LTs[32]; both LTC4[28] and LTD4[33] can interact with cys LT1R on neutrophils and induce their activation. Therefore, cys LTs might act in an autocrine and paracrine manner on cys LT1Rs present on granulocyte membranes.[31]

Synthetic LTC4 binds to human PMN receptors with rapid saturation, and its action has been found to be 90% reversible.[34] The features of these receptors are compatible with their role in mediating uptake and metabolism of LTC4 by PMNs.[34] In a study of the metabolism of cys LTs, Raulf et al[35] found that, in contrast to LT release, PMA and OPZ were more potent in activating the metabolism of LTC4 and LTD4 by PMNs compared with calcium ionophore. That study also indicated that inhibitors of oxidative burst decreased LTC4 metabolism.

We also found that zafirlukast did not inhibit superoxide generation from the X-XOD system. This finding suggests that the antioxidant effect of zafirlukast does not originate from its ability to scavenge superoxide but rather from its capacity to inhibit ROS generation in PMNs.

Based on the findings of the present study, we speculate that the activation of PMNs in patients with bronchial asthma results in increased generation of LTs, which, in turn, act on PMN cys LT1R to stimulate the release of ROSs. Our results suggest that zafirlukast interferes with this autocrine activation of PMNs by blocking the cys LT receptors, thereby inhibiting ROS production.

Our findings might explain the mechanism of action of cys LTs in producing tissue inflammation and might indicate that LT receptor antagonists could, in addition to their use as anti-inflammatory drugs, be used as antioxidants.

The present study had some limitations, including the small number of patients and the fact that they had mild to moderate stable asthma treated with β_2-agonists as needed. In addition, this was an in vitro study. It would be interesting to study asthmatic patients during exacerbations of their disease and to perform in vivo measurements to determine whether the findings are confirmed.

CONCLUSIONS

In this study of the effects of zafirlukast on PMNs in asthmatic patients and healthy controls, zafirlukast inhibited ROS production when PMNs were stimulated with PMA but not OPZ in asthmatic patients. In contrast, in healthy controls, zafirlukast inhibited ROS production by PMNs when stimulated with PMA or OPZ. Incubation of zafirlukast with PMNs for up to 1 hour increased its inhibitory effect on ROS production in healthy controls. The LT receptor antagonist zafirlukast had no scavenging effect on ROS generation in human isolated PMNs.

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REFERENCES

1. Gern JE, Lemanske RF Jr, Busse WW. Early life origins of asthma. *J Clin Invest.* 1999;104:837–843.
2. Hanazawa T, Kharitonov SA, Barnes PJ. Increased nitrotyrosine in exhaled breath condensate of patients with asthma. *Am J Respir Crit Care Med.* 2000;162:1273–1276.
3. Henson PM, Wenzel SE. Neutrophils and their mediators in asthma. In: Busse WW, Holgate ST, eds. *Asthma and Rhinitis.* 2nd ed. Oxford, United Kingdom: Blackwell Science; 2000:503–517.
4. Surette ME, Krump E, Picard S, Borgeat P. Activation of leukotriene synthesis in human neutrophils by exogenous arachidonic acid: Inhibition by adenosine A(2a) receptor agonists and crucial role of autocrine activation by leukotriene B(4). *Mol Pharmacol.* 1999;56:1055–1062.
5. Hay DW, Torphy TJ, Undem BJ. Cysteinyl leukotrienes in asthma: Old mediators up to new tricks. *Trends Pharmacol Sci.* 1995;16:304–309.
6. Menard G, Bissonnette EY. Priming of alveolar macrophages by leukotriene D(4): Potentiation of inflammation. *Am J Respir Cell Mol Biol.* 2000;23:572–577.
7. Johnson HG, McNee ML. Secretogogue responses of leukotriene C4, D4: Comparison of potency in canine trachea in vivo. *Prostaglandins.* 1983;25:237–243.
8. Marom Z, Shelhamer JH, Bach MK, et al. Slow-reacting substances, leukotrienes C4 and D4, increase the release of mucus from human airways in vitro. *Am Rev Respir Dis.* 1982;126:449–451.
9. O'Byrne PM, Israel E, Drazen JM. Antileukotrienes in the treatment of asthma. *Ann Intern Med.* 1997;127:472–480.
10. Lockhart A, Dinh-Xuan AT. Physiopathology of asthma: What role for leukotrienes? [in French]. *Rev Pneumol Clin.* 1997;53:119–127.
11. Leff JA. Leukotriene modifiers as novel therapeutics in asthma. *Clin Exp Allergy.* 1998;28(Suppl 5):147–153.

12. Weiss EB, Bellino JR. Leukotriene-associated toxic oxygen metabolites induce airway hyperreactivity. *Chest.* 1986;89:709–716.
13. Lipworth BJ. The emerging role of leukotriene antagonists in asthma therapy. *Chest.* 1999;115:313–316.
14. Edelman JM, Turpin JA, Bronsky EA, et al, for the Exercise Study Group. Oral montelukast compared with inhaled salmeterol to prevent exercise-induced bronchoconstriction. A randomized, double-blind trial. *Ann Intern Med.* 2000;132:97–104.
15. Calhoun WJ. Summary of clinical trials with zafirlukast. *Am J Respir Crit Care Med.* 1998;157(Suppl):S238–S246.
16. Busse WW, McGill KA, Horwitz RJ. Leukotriene pathway inhibitors in asthma and chronic obstructive pulmonary disease. *Clin Exp Allergy.* 1999;29:110–115.
17. Cakmak G, Demir T, Gemicioglu B, et al. The effects of add-on zafirlukast treatment to budesonide on bronchial hyperresponsiveness and serum levels of eosinophilic cationic protein and total antioxidant capacity in asthmatic patients. *Tohoku J Exp Med.* 2004;204:249–256.
18. Hasegawa H, Suzuki K, Nakaji S, Sugawara K. Analysis and assessment of the capacity of neutrophils to produce reactive oxygen species in a 96-well microplate format using lucigenin- and luminol-dependent chemiluminescence. *J Immunol Methods.* 1997;210:1–10.
19. Van Dyke K, Van Dyke C, Udeinya J, et al. A new screening system for nonsteroidal anti-inflammatory drugs based upon inhibition of chemiluminescence produced from human cells (granulocytes). *Clin Chem.* 1997;25:1655–1661.
20. Gionchetti P, Guarnieri C, Campieri M, et al. Scavenger effect of sulfasalazine, 5-aminosalicylic acid, and olsalazine on superoxide radical generation. *Dig Dis Sci.* 1991;36:174–178.
21. Braga PC, Dal Sasso M, Dal Negro R. Inhibitory effects of zafirlukast on respiratory bursts of human neutrophils. *Drugs Exp Clin Res.* 2002;28:133–145.
22. Della Bianca V, Grzeskowiak M, Dusi S, Rossi F. Transmembrane signaling pathways involved in phagocytosis and associated activation of NADPH oxidase mediated by Fc gamma Rs in human neutrophils. *J Leukoc Biol.* 1993;53:427–438.
23. Sala A, Folco G. Neutrophils, endothelial cells, and cysteinyl leukotrienes: A new approach to neutrophil-dependent inflammation? *Biochem Biophys Res Commun.* 2001;283:1003–1006.
24. Raulf M, Konig W. Modulation of leukotriene release from human polymorphonuclear leucocytes by PMA and arachidonic acid. *Immunology.* 1988;64:51–59.
25. Werz O, Klemm J, Samuelsson B, Radmark O. Phorbol ester up-regulates capacities for nuclear translocation and phosphorylation 5-lipoxygenase in Mono Mac 6 cells and human polymorphonuclear leukocytes. *Blood.* 2001;97:2487–2495.
26. Kato M, Nakano M, Morikawa A, et al. Ability of polymorphonuclear leukocytes to generate active oxygen species in children with bronchial asthma. Use of chemiluminescence probes with a Cypridina luciferin analog and luminol. *Int Arch Allergy Appl Immunol.* 1991;95:17–22.
27. Berends C, Hoekstra MO, Dijkhuizen B, et al. Expression of CD35 (CR1) and CD11b (CR3) on circulating neutrophils and eosinophils from allergic asthmatic children. *Clin Exp Allergy.* 1993;23:926–933.
28. Di Gennaro A, Carnini C, Buccellati C, et al. Cysteinyl-leukotrienes receptor activation in brain inflammatory reactions and cerebral edema formation: A role for transcellular biosynthesis of cysteinyl-leukotrienes. *FASEB J.* 2004;18:842–844.

29. Steinke JW, Borish L. Leukotriene receptors in rhinitis and sinusitis. *Curr Allergy Asthma Rep.* 2004;4:217–223.
30. Accomazzo MR, Rovati GE, Vigano T, et al. Leukotriene D4-induced activation of smooth-muscle cells from human bronchi is partly Ca2+-independent. *Am J Respir Crit Care Med.* 2001;163:266–272.
31. Figueroa DJ, Breyer RM, Defoe SK, et al. Expression of the cysteinyl leukotriene 1 receptor in normal human lung and peripheral blood leukocytes. *Am J Respir Crit Care Med.* 2001;163:226–233.
32. Minoguchi K, Adachi M. Leukotriene modifiers [in Japanese]. *Nippon Rinsho.* 2001;59: 1979–1985.
33. Bouchelouche PN, Berild D. Possible existence of leukotriene D4 receptors and mechanism of their signal transduction in human polymorphonuclear leukocytes. *Scand J Clin Lab Invest Suppl.* 1991;204:47–55.
34. Baud L, Koo CH, Goetzl EJ. Specificity and cellular distribution of human polymorphonuclear leucocyte receptors for leukotriene C4. *Immunology.* 1987;62:53–59.
35. Raulf M, Stuning M, Konig W. Effect of cations on leukotriene release: Requirements for the metabolism of peptide-leukotrienes (leukotrienes C4, D4) by human polymorphonuclear granulocytes. *Immunology.* 1986;58:479–487.

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